IN THE DRAWINGS

Replacement drawing sheets canceling Figures 6 and 7 are attached.

REMARKS

Reconsideration and allowance are respectfully requested.

Claims 1-2, 5-14, 16-17 and 23-24 are pending. The amendments are supported by the original disclosure and, thus, no new matter is added by their entry. The deleted portions of the specification correspond to canceled claims 18-21 and are not required to support the pending claims. The third paragraph on page 7 of the specification is also amended to conform with the present scope of claim 1, which is limited to testing a whole blood sample(s) comprising neutrophils. Punctuation in claims 6-8 is corrected. Claims 6-7 are also amended to cancel improper dependent clauses, and to add claims 23-24 as directed to those dependencies. Non-elected claim 22 was withdrawn from consideration by the Examiner. Applicants cancel the non-elected claim without prejudice to future prosecution of that subject matter.

Part (b) of claim 1 clarifies the time period at which superoxide production above basal in the test whole blood sample is determined following addition of the inducer (e.g., PMA). This must be the time period at which the same inducer under the same conditions induces superoxide production above basal (i.e., minus inducer) in the control whole blood sample. A suitable control sample will be whole blood from an individual from the same species in which the neutrophils "are free or substantially free of stress-induced activation or at least derived from one or more individuals exposed to the same regime minus a factor to be tested as a psychological stressor."

35 U.S.C. 112 – Definiteness

Claims 1-14 and 16-21 were rejected under Section 112, second paragraph, as being allegedly "indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention." Applicants traverse.

Claim 1 is amended to correct antecedent basis issues raised by the Examiner, which does not affect the scope of the claims. In addition the definitions of "time period" between induction and determining superoxide production, "test" sample, and "control" samples are clarified.

Applicants request withdrawal of the Section 112, second paragraph, rejection because the pending claims are clear and definite.

Applicants' Invention and Examiner's Comments Thereon

In claim 1, the finding of **lower** superoxide production above basal in the test whole blood sample in the presence of inducer as compared to superoxide production above basal in the control whole blood sample in the presence of inducer is a marker of a stressful event. Psychological stress in itself causes neutrophil activation with consequent superoxide production. In an individual affected by a stressful event, it is possible to look for retained capacity of neutrophils to produce superoxide in the presence of an added inducer such as PMA by a simple whole blood test whereby lower induced superoxide production in the test sample compared to the control sample is an immediate indicator of the effect of a psychological stressor. Where lower superoxide production is found in the test sample, the extent to which the inducer stimulates superoxide production is a measure of coping capacity, i.e., this is an objective measure of exposure to stress in terms of its effect on neutrophils. Little or no induced superoxide production in the test sample indicates that the neutrophils have been exhausted, or almost exhausted, of capability for superoxide production by exposure to the stressful event.

Claim 2 addresses the situation where an individual is exposed to a psychological stressor whereby the neutrophils in the whole blood test sample are dampened in ability to produce superoxide in response to challenge by an inducer, e.g., PMA, and the degree of further *in vitro* induced superoxide production in the test sample above basal is used as an objective measure of coping capacity for the stress exposure of concern. This may be, for example, handling and/or transport in the case of animals as illustrated by Examples 1 and 2. It may be any of the many stress factors pertinent to daily life of humans ranging from horror film viewing (Example 3), mental tests (Example 4), performance stress arising from sporting activities (Example 5), medical treatments, and environmental factors. Claim 2 is amended to emphasize the character of the test for coping capacity consistent with the amended ending of claim 1.

From the comments of the Examiner, it would seem that she sees the invention as somehow related to a change in leucocyte/neutrophil number and, in particular, stress leading to the ability to detect increased numbers of such cells through superoxide production. This is simply not correct. It is again emphasized that claim 1 refers to detection of **lower** induced superoxide production above basal in test samples taken from individuals affected by a psychological stressor. While the Kang et al. and Morrow-Tesch et al. documents do refer to elevated numbers of leucocytes/neutrophils associated with stress, subsequent studies have shown that this is not always to be expected and more importantly PMA-induced chemiluminescence in blood samples does not in any case correlate with neutrophil number (although a range of leucocytes can produce superoxide, neutrophils are responsible for the vast majority of superoxide production).

Examples 1 to 5 of Applicants' specification illustrate consistently lower superoxide measurement by chemiluminescence assay in test whole blood samples above basal following PMA-induced challenge compared with superoxide production above basal in control whole blood samples where the test samples are taken from individuals subjected to a stress factor. Lower PMA-induced superoxide production in test samples above basal (in the absence of inducer) is a consistent finding requiring no reference to actual neutrophil numbers. As will be expanded upon further below, it is submitted that none of the cited documents points to the feasibility of such a convenient blood test for quantification of stress exposure.

It is to be noted that in the badger studies reported in Example 1, leucocyte numbers in samples were determined and total superoxide production was measured as superoxide production per 10^9 neutrophils/l. Differences in leucocyte numbers were found between samples from the test animals (transported) and the control animals (not transported), with transported individuals having a higher percentage of neutrophils. Nevertheless whether leucocyte coping capacity was calculated per 10^9 neutrophils/l or equated with chemiluminescence assay of total superoxide production above basal (without inducer), the same pattern of results was observed: uniform depression of superoxide production in response to PMA challenge (also sometimes referred to as PMA-induced *in vitro* respiratory burst) in test samples from transported animals

compared to control samples from non-transported animals (see the 2nd and 3rd paragraphs on page 18 and Figs. 1-2 of Applicants' specification). The same study is reported and discussed in the publication of the inventors McLaren et al. Exp. Physiol. 88:154-156 (2003), a copy of which is provided. This publication further emphasizes the novel finding underpinning the invention and its importance for enabling rapid objective assessment of coping capacity for any stressful experience in the field or laboratory by merely carrying out a chemiluminescence assay on whole blood samples.

The Examiner is also referred to another publication by Honess et al. Animal Welfare 14:291-295 (2005), a copy of which is provided, which reports later successful application of the invention to non-human primates, more particularly to look at the difference in stress in a breeding colony of rhesus macaques depending on housing conditions (traditional caging versus open-rooms). In this instance, no difference was found between the two groups in leucocyte numbers or composition (see abstract). But a PMA-challenge test in accordance with the invention showed that blood samples obtained by venepuncture from caged animals produced a significantly lowered induced superoxide production indicative of caging being a stress factor compared with open-room housing.

Such studies further verify that the invention is unrelated to changes in leucocyte/ neutrophil number and represents an important tool for quantifying stress which cannot be deduced directly. In the International Preliminary Examination Report for the parent PCT application, it was concluded that "the prior art does not show that said stress exposure leads to **less** superoxide production *in vitro* after stimulation by an inducer compared to an unexposed control" (Section V. 4). This conclusion is not affected by the documents newly cited in this instance as now expanded upon with reference to the specific rejections under 35 U.S.C. § 102 and 35 U.S.C. § 103. Moreover, it is submitted that no other document of record either teaches or even suggests extrapolation of this concept in relation to whole blood samples.

35 U.S.C. 102 – Novelty

A claim is anticipated only if each and every limitation as set forth in the claim is found, either expressly or inherently described, in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of Calif.*, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). The identical invention must be shown in as complete detail as is claimed. See *Richardson v. Suzuki Motor Co.*, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989).

Claims 1-5, 9 and 18-20 were rejected under Section 102(b) as allegedly anticipated by Kang et al. (Brain Behav. Immun. 10:164-181, 1996). Applicants traverse.

The Kang et al. document reports an increase rather than a decrease in superoxide production by neutrophils in response to PMA-challenge post exams in healthy adolescents and asthmatics (only some asthmatics showed what is termed a surprising reduction in superoxide production with FMLP challenge) (see the 1st paragraph under Table 1 on page 170). Hence, the Kang et al. document presents results which are very different from those to be expected from practicing Applicants' invention as set out in their specification (see particularly Example 4). The PMA/FMLP-challenge assays described in the Kang et al. document are also significantly different from Applicants' invention.

It is an important feature of the invention that whole blood samples are used with a view to maintaining the responsiveness of the leucocytes as *in vivo*. In contrast, the Kang et al. document employed for its PMA/FMLP-challenge assays isolated neutrophils subjected to a lengthy handling procedure: density gradient centrifugation, re-suspension without Ca^{2+} and Mg^{2+}, mixing with dextran and maintenance at room temperature for one hour, harvesting of the neutrophil-rich supernatant, lysis of red blood cells with sterile water, washing with Hank's buffered saline solution (HBSS) minus Ca^{2+} and Mg^{2}, and finally re-suspension at a particular concentration of cells using HBSS with Ca^{2+} and Mg^{2+} (see the bottom two paragraphs on page 166 under the heading "*Procedures*" in the Kang et al. document). The use of isolated neutrophils obtained in this manner meant that superoxide production could be measured by colorimetric assay (reduction of ferricytochrome C; see assay description on page 167), but also meant that the neutrophils were subject to conditions liable to affect responsive-

ness to PMA. Significantly, the response to PMA is calcium dependent, and therefore removing and adding calcium is one factor liable to affect subsequent reactivity. It is thus hardly surprising that the results of the PMA-challenge assays of the Kang et al. document are at variance with the results presented in the subject application.

The Kang et al. document thus neither anticipates nor renders obvious Applicants' invention; nor does it contradict the results obtained by Applicants. It fails entirely to teach or suggest the concept underpinning the invention because the prior art focused on observing an increase of leucocyte numbers in blood samples from exam candidates during and after exams, and the authors' use of isolated neutrophils for induced super-oxide production release.

Withdrawal of the Section 102 rejection is requested because all limitations of the claimed invention are not disclosed by the cited document.

35 U.S.C. 103 – Nonobviousness

To establish a case of prima facie obviousness, all of the claim limitations must be taught or suggested by the prior art. See M.P.E.P. § 2143.03. Obviousness can only be established by combining or modifying the prior art teachings to produce the claimed invention if there is some teaching, suggestion, or motivation to do so found in either the references themselves or in the knowledge generally available to a person of ordinary skill in the art. See, e.g., *In re Fine*, 5 USPQ2d 1596, 1598 (Fed. Cir. 1988); *In re Jones*, 21 USPQ2d 1941, 1943-44 (Fed. Cir. 1992). A determination of prima facie obviousness requires a reason-able expectation of success. See *In re Rinehart*, 189 USPQ 143, 148 (C.C.P.A. 1976).

Claims 1 and 6-8 were rejected under Section 103(a) as allegedly unpatentable over Kang et al. (Brain Behav. Immun. 10:164-181, 1996) in view of Morrow-Tesch et al. (J. Anim. Sci. 72:2599-2609, 1994). Applicants traverse.

Combining the Kang et al. and Morrow-Tesch et al. documents is of no greater assistance in suggesting Applicants' invention. The observation that neutrophils are elevated in pigs subject to stressful housing conditions, like the observation in the Kang et al. document that exam stress can cause elevated neutrophils in humans, is no moti-

vation whatsoever for one of ordinary skill in the art to look to observation of **lowered** superoxide production in whole blood samples in response to an inducer (e.g., PMA challenge) as a stress marker as required by Applicants' claims.

Claims 1-5 and 9-1 were rejected under Section 103(a) as allegedly unpatentable over Kang et al. (Brain Behav. Immun. 10:164-181, 1996) in view of Pfefferkorn et al. (U.S. Patent 5,492,816). Applicants traverse.

The Pfefferkorn patent merely provides enhanced chemiluminescence assays for superoxide. While such an assay might be employed in substitution for the colorimetric assay employed in the Kang et al. document, this is insufficient grounds to arrive at Applicants' invention, which depends on an entirely different premise for assessing stress exposure: i.e., a premise arising from consideration of neutrophil responsiveness to inducer (e.g., PMA) rather than neutrophil numbers.

Claims 1-5, 12-14 and 16-21 were rejected under Section 103(a) as allegedly unpatentable over Kang et al. (Brain Behav. Immun. 10:164-181, 1996) in view of Morrow-Tesch et al. (J. Anim. Sci. 72:2599-2609, 1994), and further in view of Ellard et al. (Intl. J. Psycho. 41:93-100, 2001). Applicants traverse.

The Examiner is correct in characterizing the Ellard et al. document as disclosing that a short mental stress will increase neutrophil activation. Indeed, this document was cited for this reason in the background section of Applicants' specification (see 2nd paragraph on page 2). It provides no basis, however, for extrapolating to the method of Applicants' invention as a workable concept. The only neutrophil studies reported in the Ellard et al. document relied on use of nitroblue tetrazolium (NBT) to stain isolated activated neutrophils on microscope slides. Such assays visualize the formation of formazon granules and provide no information relevant to relying on PMA challenge and superoxide measurement in whole blood samples as a means of quantifying stress. This only became apparent as a workable concept with the carrying out of the studies reported by Applicants. The cited document fails to provide a reasonable expectation of success to combine the Kang et al. and Morrow-Tesch et al. documents in the manner proposed by the Examiner.

Withdrawal of the Section 103 rejections is requested because the invention as claimed would not have been obvious to one of ordinary skill in the art at the time it was made.

Conclusion

Having fully responded to all of the pending rejections contained in this Office Action, Applicants submit that the claims are in condition for allowance and earnestly solicit an early Notice to that effect. The Examiner is invited to contact the undersigned if any further information is required.

Respectfully submitted,

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Experimental Physiology

Leukocyte coping capacity: a novel technique for measuring the stress response in vertebrates

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Methods used to quantify the stress response in animals are vital tools in many areas of biology. Here we describe a new method of measuring the stress response, which provides rapid results and can be used in the field or laboratory. After a stressful event, we measure the capacity of circulating leukocytes to produce a respiratory burst *in vitro* in response to challenge by phorbol myristate acetate (PMA). During the respiratory burst leukocytes produce oxygen free radicals, and the level of production can be measured directly as chemiluminescence. When *in vitro* PMA-stimulated whole blood chemiluminescence is measured directly after a stressful event, we define the response as the leukocyte coping capacity (LCC). In an experiment badgers (*Meles meles*), which were caught as part of an on-going population study, were either transported to a central site prior to blood sampling or blood was collected at their site of capture. Transported animals had a significantly lower LCC and showed changes in leukocyte composition that were indicative of stress. We conclude that the stress of transport reduced LCC in badgers and that LCC serves as a quantitative measure of stress. Potential applications of this method are discussed. *Experimental Physiology* (2003) **88.4**, 541–546.

Objective, quantitative and practicable measures of stress are pivotal to studies in many branches of vertebrate biology, including human biology, animal husbandry and wildlife ecology (e.g. Dawkins, 1980; Bateson & Bradshaw, 1997; Palme & Möstl, 1997; Creel, 2001; Goymann *et al.* 2001). The stress response in animals is currently assessed using a variety of techniques, including measurement of cortisol levels (e.g. Beerda *et al.* 1996; Palme & Möstl, 1997; Harper & Austad, 2000) and haematological values (e.g. Millspaugh *et al.* 2000), and observations of behaviour (reviewed by Rushen, 2000). Here we present a new method for quantifying stress, based on direct measures of an immune response. We state the benefits of the technique, and discuss the circumstances under which its use is most valuable.

The method is based on the ability of individuals to mount a challenge-induced immune response after a defined, potentially stressful event. Each individual's capacity to respond to immune challenge is compared with its own baseline level of immune system activity. After the putatively stressful event, we measure the capacity of the individual's leukocytes to produce a quantifiable immune response known as the respiratory burst. During the respiratory burst, oxygen uptake by leukocytes is accelerated in order to produce oxygen free radicals that destroy bacteria (a process reviewed by Halliwell & Gutteridge, 2000). Leukocytes are known to produce oxygen free radicals in response to agonists such as bacterial peptides binding to receptors on their cell membranes (Dietert *et al.* 1996), the activation of protein kinase C with phorbol myristate acetate (PMA; Hu *et al.* 1999) and stress (Ellard *et al.* 2001). It has also been demonstrated that stress affects the respiratory burst: leukocytes isolated from the head kidney of salmon (*Salmo salar*) showed a reduced respiratory burst (40 % reduction in oxygen free radical production) after the fish were subjected to a 2 h period of confinement stress (Thompson *et al.* 1993). Therefore, there is evidence that the respiratory burst activity of leukocytes is affected by stress. In particular we wanted to determine whether a reduction in the respiratory burst of circulating leukocytes (which can be measured in whole blood without using isolation techniques) could be used as a measure of stress. In our *in vitro* challenge–coping approach, after a stressful event, we chemically stimulate a respiratory burst in whole blood *in vitro* using PMA, and measure the capacity of

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leukocytes to produce a respiratory burst over a 30 min period.

We define the response of leukocytes to PMA challenge after a stressful event as the individual's leukocyte coping capacity (LCC). Therefore, animals with a higher LCC have a greater potential to produce a respiratory burst, and physiologically, are better able to respond to bacterial challenge after stress. Therefore LCC is an *in vitro* assessment of the animal's current physiological status. We also examine the basal chemiluminescence in samples of blood that have not been stimulated by PMA, and this acts as a baseline with which to compare the individual's LCC. Our hypothesis is that LCC, measured using a challenge–coping approach, will provide a quantitative measure of the stress an individual undergoes during a particular event. This hypothesis predicts that animals subjected to known stressors should have a lower LCC than animals that have not (Sanidas *et al.* 2000; Egger *et al.* 2001; Ostrakhovitch & Afanas'ev, 2001). Here, we tested the prediction that badgers (*Meles meles*) have a lower LCC when they are subjected to trapping and then transport, a known stressor in animals (Blecha, 2000) than when they are trapped but do not experience the additional stress of transport.

METHODS

Trapping and transporting badgers

Badgers were trapped in Wytham Woods, Oxfordshire, UK (for details of the study site and its badger population see Macdonald & Newman, 2002) in cage traps baited with peanuts in August and November 2001. We selected badgers because a measure of stress for this species would be immediately useful in studies of its unusual social system (Macdonald & Newman, 2002), its role in the epidemiology of bovine tuberculosis and because studies, conservation and control of badgers necessitate their capture and handling, so a means of evaluating alternative procedures would be helpful. We selected this population because their individual life histories have been monitored for 14 years, during which our particular trapping and handling procedures have been refined to the highest welfare standards. Badger traps were set adjacent to badger setts between 14.00 and 17.00 h. Traps were checked each morning between 06.30 and 07.00 h and trapped badgers were transferred to individual holding cages, which were then covered.

Animals were then assigned to one of two experimental regimes: sampling at the site of capture, without transport (non-transported, $n = 8$); or sampling immediately after transport (transported, $n = 8$). Transport consisted of a short ride for less than 10 min on a trailer pulled by an all-terrain quad bike. Badgers were anaesthetised, either at their site of capture or after transport, using an intramuscular injection of ketamine hydrochloride at a concentration of 100 mg ml^{-1} (Ketaset, Fort Dodge, USA) and administered to badgers at a dose of 0.2 ml kg^{-1}. Processing consisted of measurements of body weight and length, and recording the sex and condition of the badger. Other measurements were also taken as part of the ongoing badger population study. Blood was removed by needle venepuncture of the jugular vein, collected into a tube containing the anti-coagulant potassium EDTA (BD Vucutainer Systems, Plymouth, UK) and taken immediately for PMA challenge. Differential cell counts were made from blood smears fixed in alcohol and stained with May-Grunwald and Giemsa stains (minimum of 100 cells counted per slide). Mean cell volume (MCV), mean cell haemoglobin concentration (MCHC) and haematocrit were calculated using a haematology analyser (Celltac MEK-5108K, Kohden, Japan).

PMA challenge and measurement of leukocyte coping capacity

To measure the unstimulated blood chemiluminescence levels, 10 μl whole blood was transferred into a silicon anti-reflective tube (Lumivial, EG & G Berthold, Germany) to which 90 μl 10^{-4} mol l^{-1} luminol (5-amino-2,3-dihydrophthalzine; Sigma A8511) diluted in phosphate buffer was added. The tube was then shaken gently. To measure the chemiluminescence produced in response to challenge, a further tube was prepared as above, but with the addition of 10 μl phorbol 12-myristate 13-acetate (PMA; Sigma P8139) at a concentration of 10^{-6} mol l^{-1}. The PMA was dissolved in a small amount of dimethyl sulfoxide (DMSO; Sigma D 5879) and then diluted to a concentration of 10^{-6} mol l^{-1} in PBS. For each tube chemiluminescence was measured every 5 min in a portable chemiluminometer (Junior LB 9509, EG & G Berthold) for a total of 30 min. When not in the chemiluminometer, tubes were incubated at 37 °C. Although a range of leukocytes can produce a respiratory burst, neutrophils are responsible for the majority of oxygen free radical production (Ellard *et al.* 2001) and so we also examined LCC per quantity of 10^9 neutrophils l^{-1}; this also provided a method of examining LCC in relation to the potential effects of changes in the number of circulating neutrophils after stress.

Statistical analyses

To compare differences in LCC and other haematological parameters in badgers with and without transport, we used multivariate analysis of variance (MANOVA). For LCC, the chemiluminescence levels at each time interval were the dependent variables. We chose this approach because it allowed us to examine the effect of the treatment at each time interval, giving more detail about the nature of the effects of the treatment, and allowed us to identify the time interval at which the treatment effect was greatest. This procedure was carried out using the software SPSS for Windows release 10.0.5. Non-parametric data were log transformed prior to the analysis to ensure the data met assumptions of multivariate normality (Tabachnick & Fidell, 1996).

Animal welfare considerations

The badger population at Wytham Woods is under long-term scientific investigation, and badgers are trapped and transported regularly throughout their lives. Examining transport stress is part of our continuing refinement of best practice. Work was carried out under English Nature licence 1991537 and UK Home Office licence PPL 30/1826.

RESULTS

LCC and unstimulated blood chemiluminescence levels for transported ($n = 8$) and non-transported ($n = 8$) badgers are shown in Fig. 1. Transport significantly reduced LCC in badgers ($F_{7,8} = 4.5$, $P = 0.03$). The difference in LCC between transported and non-transported individuals was greatest at 15 min ($F_{1,14} = 8.9$, $P = 0.01$; Fig. 1). However, two of the non-transported individuals were caught during a night when ambient temperatures unexpectedly fell below freezing, and these individuals did not show a

typical response for this group, and in both cases their LCC was no greater than their basal response (Wilcoxon Signed Ranks Test, for both cases $Z < -1.0$, $P > 0.1$). Transport had no effect on unstimulated levels of chemiluminescence ($F_{7,8} = 2.5$, $P > 0.05$). However, to ensure that there was no bias in the LCC results because of individual differences in unstimulated levels of chemiluminescence, we subtracted the unstimulated from the PMA-stimulated values for each animal (PMA – unstimulated). Transport had an effect on the PMA – unstimulated values from 10 min ($F_{1,14} = 4.1$, $P = 0.06$), with the difference peaking at 15 min ($F_{1,14} = 9.4$, $P = 0.008$), and thus showed a pattern consistent with the LCC values described above. To examine individual neutrophil activity, we calculated LCC per 10^9 neutrophils l^{-1} (Fig. 1), and found the same pattern as overall LCC, indicating that activity per neutrophil was greater in non-transported individuals.

The effects of transport on the blood parameters we measured are shown in Table 1. Transported individuals had significantly lower numbers of leukocytes and a different leukocyte composition: transported animals had a higher percentage of neutrophils and a correspondingly lower percentage of lymphocytes (Table 1). Haematocrit, neutrophil/lymphocyte ratio, MCHC and MCV were also affected by transport (Table 1).

DISCUSSION

These results indicate that stress dramatically reduces the potential of circulating leukocytes to produce free radicals, and moreover, the results support the hypothesis that LCC is a measure of the stress associated with a specific event. The rather uniform depression of the coping response in all transported individuals suggests that this stressor overrode the sources of individual variation that characterised the coping responses of non-transported individuals. The reduced response of the two individuals caught during a night when temperatures fell below freezing perhaps identifies another stressor worthy of investigation (Glette *et al.* 1982).

Transport also brought about changes in circulating cell composition and number, and this represents the effects of cell trafficking between reservoir sites – the liver, lungs, bone marrow and peripheral blood (Severs *et al.* 1996). In particular, the increases in haematocrit and neutrophil/lymphocyte ratio in the transported animals are indicative of stress (Murata, 1989; Haigh *et al.* 1997; Maes *et al.* 1998). Stress hormones are known to bring about changes in circulating leukocyte composition (McCarthy & Dale, 1988). Immediate leukocytosis is attributed to elevated catecholamine levels, and delayed increases in neutrophils and decreases in lymphocytes are observed due to raised

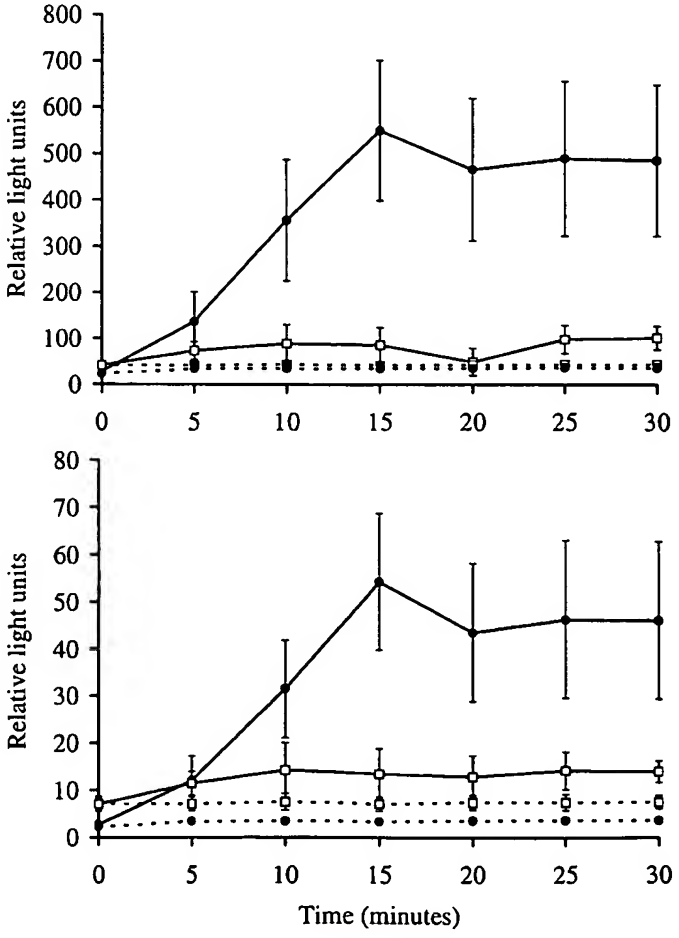

Figure 1

The effect of transport stress on *in vivo* leukocyte coping capacity (LCC). The continuous lines represent mean (± S.E.M.) LCC in badgers after transport (□), and without transport (●). Dashed lines represent unstimulated control samples for transported (□), and non-transported (●) badgers. The lower graph presents LCC per neutrophil, where LCC has been calculated as relative light units divided by the number of neutrophils ($10^9 l^{-1}$). Lines and symbols are as in upper graph.

Table 1. Blood parameters measured in transported and non-transported animals

	Non-transported		Transported				
	Mean	S.E.M.	Mean	S.E.M.	t	d.f.	P
Erythrocytes ($\times 10^{12}$ l^{-1})	7.1	0.7	9.8	0.8	−2.5	14	0.025
Haemoglobin (g l^{-1})	113.8	10.1	144.4	11.5	−2.0	14	0.065
Haematocrit (%)	34.0	3.1	44.5	3.3	−2.3	14	0.037
MCV (fl)	48.3	0.6	45.8	0.8	2.6	14	0.020
MCH (pg)	16.2	0.2	14.8	0.2	4.7	14	0.000
MCHC (g l^{-1})	335.4	2.6	324.1	3.0	2.9	14	0.013
Platelets ($\times 10^{9}$ l^{-1})	618.3	87.3	525.5	49.1	0.9	14	0.370
Leukocytes ($\times 10^{9}$ l^{-1})	10.4	0.9	7.0	0.9	2.5	14	0.024
Neutrophils (%)	81.5	2.4	88.5	2.1	−2.2	14	0.047
Lymphocytes (%)	17.4	2.4	10.4	2.2	2.1	14	0.052
Monocytes (%)	1.1	0.5	1.1	0.6	0.0	14	0.999
Eosinophils (%)	0.0	0.0	0.0	0.0	—	—	—
Basophils (%)	0.0	0.0	0.0	0.0	—	—	—
Neutrophils ($\times 10^{9}$ l^{-1})	8.5	0.9	6.3	0.9	1.8	14	0.098
Neutrophil/lymphocyte ratio	5.3	0.7	10.7	1.6	−3.0	14	0.009

Mean and standard error of the mean (S.E.M.) are presented for both treatment groups (n = 8 for both), and the significance of the differences between the groups are reported based on a t test, for which the degrees of freedom (d.f.) and significance (P) are given. MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration.

cortisol levels (Pedersen *et al.* 1994). It is therefore probable that in badgers subjected to transport, increased cortisol levels brought about the changes we observed in the neutrophil/lymphocyte ratio.

Transported badgers had lower numbers of circulating leukocytes. In contrast, short-term stress is also usually associated with a demargination of leukocytes (entry into the circulation): a relationship we have observed in our laboratory (Mian *et al.* 2003). However, this relationship is not simple (Dhabhar *et al.* 1996), and in an elegant series of experiments Oishi *et al.* (2003) demonstrated that immune changes in rats brought about by physical stimulators differed from those induced by visual, olfactory and auditory stimuli. Modification of the receptors on the endothelium and on the leukocytes themselves can dramatically alter the number of adherent (and thus the number of circulating) leukocytes (Ley, 1996). Transport stress could therefore have modified these receptors and caused an increased margination of the leukocytes. Margination is the process by which leukocytes exit the central blood stream, and initiate mechanical contact with the endothelial cells. The margination process is enhanced in vessels of a certain size by the aggregation of erythrocytes, which tend to occupy the centre of microvessels (Firrell & Lipowsky, 1989). The increase in haematocrit observed in this and previous studies (Maes *et al.* 1998) could thus selectively promote margination in some vessels. Previous studies have demonstrated that margination of leukocytes is not a uniform process, and occurs in particular sized vessels within the microcirculation (Mian & Marshall, 1993). The changes in shear stress likely to have been brought about by the increased haematocrit may also serve as a trigger mechanism for leukocyte activation (Schmid-Schonbeim *et al.* 2001). It has been suggested that exposure to hostile conditions, or other psychological stressors, initiates the secretion of several hormones including cortisol, catecholamines, prolactin, oxytocin and renin (Toft *et al.* 1994; Van de Kar & Blair, 1999). Any of these could alter adhesion receptors on circulating leukocytes and thus contribute to an altered leukocyte distribution.

Furthermore, psychological stress alone has been shown to influence the number and distribution of leukocytes in the blood in a rapid and reversible manner (Dhabhar *et al.* 1995, 1996; Kang *et al.* 1996; Goebel & Mills, 2000), and stress has also been found to alter the expression of leukocyte adhesion receptors (Goebel & Mills, 2000). Although an alteration of cell numbers and adhesion molecules is not always associated with an alteration in cellular activity and function (Mian & Marshall, 1993), it is clear that leukocytes can respond rapidly to a wide range of physical and psychological stressors, and that these responses can affect the ability of the immune system to respond to ongoing or potential challenge (Dhabhar *et al.* 1995; Gleeson & Bishop, 2000). As a result, we argue that measures of stress based on leukocyte function, such as LCC, can reveal significant information about the animal's physiological status after and during stressful events.

The relationship between LCC and other components of the stress response should be further examined, but immune function during stress is a well-studied area that provides a background to further investigation of LCC. The mechanism by which the brain modulates the immune system involves the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (Neveu,

2003). Measurements of the status of the HPA axis, in particular the production of cortisol, provide important data on the stress response. However, the magnitude of the HPA response depends on basal hormonal concentration (Milde *et al.* 2003), and cortisol levels can vary widely between individuals, obscuring the effects of stress (e.g. Montané *et al.* 2002). The immune system also plays a wide-ranging role in the stress response – for example, cytokines produced by the immune system during stress may serve as mediators of an afferent pathway to the brain and could in turn induce changes in behaviour (Buller, 2003). We have reported that immune alterations induced by stress are concomitant with increases in blood pressure and heart rate, but that the immune alterations remained long after these traditional measures of stress had returned to basal values (Mian *et al.* 2003). The most appropriate measure of stress, for a given situation, will depend upon the nature and intensity of the stressor, the experimental design and a host of other factors. We argue that stress measures based on immune system alterations are valid alternatives to measures based on the HPA axis, and may be more suitable in certain circumstances.

We have demonstrated that LCC is affected directly and rapidly by stress. The strengths of our technique, as revealed by our experiment, include: (1) the ability to take measurements during or immediately after a stressful event; (2) the direct comparability of individual responses, which themselves represent physiologically meaningful measures; and (3) the ability to take measurements in the field on whole blood (avoiding centrifugation), yielding results within minutes and without requiring baseline data from animals which have not been stressed. This method would therefore be of particular use in situations where, after or during a stressful event, a rapid assessment of the individual's ability to cope is required. In our case, LCC provided results whilst the animal was under anaesthesia. Thus, in such cases, if an individual showed an unusual response, appropriate steps could be taken well before the animal has been returned to the wild. This was demonstrated by the reduced LCC of the two individuals caught during cold conditions, indicating that, whatever the cause, these individuals had coped less well with capture than other non-transported individuals. Furthermore, the PMA challenge we present *in vitro*, is different from *in vivo* challenges such as the ACTH challenge (e.g. Goddard *et al.* 1994), because PMA challenge is a measure of the ability of the animal to respond to bacterial challenge after stress. Therefore LCC does not represent a further challenge to the individual *per se*, but is instead a measure of the animal's physiological status after a stressful event.

A rapid assessment of individual responses to the same stressor could have many other practical applications in biology, veterinary science and animal husbandry. In addition, this technique could be used in conjunction with other measures, such as faecal cortisol measurement, to indicate patterns of stress and coping in free-living vertebrates. One practical motive for our work is to disentangle the stressfulness of each component of our field procedures, so that they may be improved. This first step demonstrates the power of the coping–challenge technique. However, while we have shown that within the context of a system already carefully designed to prioritise welfare, transportation is, unsurprisingly, more stressful than no transportation, we have yet to put the magnitude of that stress into the context of the diversity of other stressors encountered by badgers either in their natural lives or during alternative handling protocols. It is exciting that our technique makes this calibration feasible, not merely in the special case of our study, but across a wide field of biological, agricultural, veterinary and medical applications.

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Assessment of stress in non-human primates: application of the neutrophil activation test

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Abstract

A technique measuring leukocyte (neutrophil) activity was used to examine differences between stress levels in a breeding colony of rhesus macaques housed in either a traditional caging system or open-rooms. The leukocyte activation test measured the degree to which blood from the two treatment groups could launch a further neutrophil response (superoxide production) to an in vitro *challenge. Animals housed in a traditional caging system produced a significantly lower leukocyte response than animals housed in open-rooms, indicating that there was a higher level of stress associated with caged housing than open-room housing. This was not influenced by whether animals were physically restrained or trained to stand for a sedating injection. No differences were found between treatment groups in leukocyte numbers or composition. This study validates the use of the leukocyte activation test to assess physiological stress levels in non-human primates and demonstrates the animal welfare benefits of open-room housing over traditional laboratory caging systems.*

Keywords: *animal welfare, housing refinement, leukocyte activity,* Macaca mulatta, *rhesus macaques, stress*

Introduction

Caging and stress

A considerable body of literature exists on the influence of housing conditions on the physiological measures of primate stress (eg Crockett *et al* 2000; Mendoza *et al* 2000; Honess & Marin 2005). Research demonstrates that stress levels cause changes that affect a number of biological functions, including immune competence (Moberg 1985; Raberg *et al* 1998; Maule & VanderKooi 1999), brain structure and function (Uno *et al* 1989; Sapolsky 1996, 2003), and reproduction (Carlstead & Shepherdson 1994; Pottinger 1999; Ha *et al* 2000).

There is a clear effect of overall cage size (eg Draper & Bernstein 1963; Paulk *et al* 1977), and its structural complexity (eg Reinhardt 1996; Röder & Timmermans 2002; Buchanan-Smith *et al* 2004; Wolfensohn & Honess 2005), on the psychological well-being of primates. An increase in space and its enrichment allows increases in socialisation and creates opportunities for training animals to cooperate with husbandry and veterinary procedures (Wolfensohn 2004). This has clear implications for the maintenance of captive animals for research and for breeding.

Measurement of stress

Traditional measurements of physiological stress have focused on the detection of cortisol levels in the blood plasma, faeces, urine or saliva (Mendoza *et al* 2000; Theorell 2003), or on monitoring autonomic responses, such as changes in heart rate or blood pressure (Porges 1985; Line *et al* 1989a,b). The implantation of telemetry devices to measure cardiovascular parameters may compound and confound stress measures, and less invasive methods of monitoring are ethically more acceptable. However, the collection of faeces, urine or saliva samples for cortisol assay is practically complex, particularly from group-housed animals on a forage substrate. Furthermore, the interpretation of cortisol assay results is complicated by considerable individual variation (Montane *et al* 2002), a natural circadian variation in cortisol levels (Sousa & Ziegler 1998; Mendoza *et al* 2000; Theorell 2003), the fact that a cortisol response is associated with some non-stress stimuli, and that some stress responses may not involve elevated cortisol levels (Moberg 2000). Although low concentrations of cortisol are typically associated with low stress, chronically stressed primates are known to exhibit hypocorticolism (Mendoza *et al* 2000). These problems have contributed to an increasing dissatisfaction with the use of cortisol to measure stress levels (Crockett *et al* 2000; Moberg 2000).

This study monitors the activity of leukocytes, which have been shown to increase oxygen uptake in response to bacterial challenge in order to produce oxygen free radicals that kill bacteria (Hu *et al* 1999; Halliwell & Gutteridge 2000). Psychological stress produces the same response (Ellard *et al* 2001) and can also influence the number and distribution of leucocytes, and the expression of their

Table 1 Analysis of leukocyte activity (relative light units [RLUs]) in rhesus macaques kept in a traditional caging system versus open-room housing conditions. Figures in brackets are one standard error, ns = non significant, ** = $P < 0.01$.

	Leukocyte activity			Leukocyte numbers and composition			
	PMA 10^{-3} mol l^{-1}	PMA 10^{-5} mol l^{-1}	Unchallenged	Leukocytes 10^9 l^{-1}	% Neutrophils	% Lymphocytes	Neutrophil-lymphocyte ratio
Caged (n = 10)	7960.68 (1178.46)	2069.49 (400.33)	1923.25 (402.11)	9.76 (1.32)	58.30 (6.68)	40.50 (6.51)	2.48 (0.77)
Open-room (n = 43)	16157.11 (2840.28)	3258.02 (525.09)	1605.92 (150.86)	9.38 (0.55)	62.47 (2.43)	36.37 (2.40)	2.28 (0.24)
Mann-Whitney *U*	97.00	184.00	190.00	215.00	186.50	185.00	185.00
P value (2-tailed)	0.007**	0.481 ns	0.570 ns	1.000 ns	0.517 ns	0.495 ns	0.495 ns

Table 2 Analysis of the effect of capture technique (standing for injection versus being netted) on leukocyte activity (RLUs) in rhesus macaques housed in open-rooms. Figures in brackets are one standard error, ns = non significant.

	PMA 10^{-3} mol l^{-1}	PMA 10^{-5} mol l^{-1}	Unchallenged
Standing (n = 13)	24129.67 (8933.49)	3473.01 (773.25)	1759.42 (301.11)
Netted (n = 22)	13766.96 (1219.71)	3856.59 (884.81)	1668.72 (222.37)
Mann-Whitney *U*	136.0	134.0	131.0
P value (2-tailed)	0.811 ns	0.759 ns	0.682 ns

adhesion receptors (Dhabhar *et al* 1995; Kang *et al* 1996; Goebel & Mills 2000). While changes in cell numbers and adhesion molecules are not exclusively associated with altered cellular activity and function (Mian & Marshall 1993), an extensive range of physical and psychological stressors do produce a rapid response in leucocytes that can affect the immune system's ability to cope with an ongoing or potential challenge (Dhabhar *et al* 1995).

This technique has already been demonstrated as an appropriate measurement of psychological stress in humans (Ellard *et al* 2001; Mian *et al* 2003) and in wild badgers in the field (McLaren *et al* 2003; Montes *et al* 2004). This study aims to examine its use in non-human primates by investigating the stress levels of rhesus macaques (*Macaca mulatta*) housed in different conditions (a traditional caging system versus open-rooms) and the extent to which any difference in stress levels is affected by capture method (physical restraint versus trained standing for injection).

Materials and methods

Study subjects

The animals used in this study were part of the Oxford University rhesus macaque breeding colony housed at Harlan, UK. This specific-pathogen-free colony consisted of approximately 200 monkeys including 16 single male breeding groups of 4–11 females and their unweaned infants, which were transferred to peer groups at 12–18 months. All macaques were group-housed indoors in either a traditional caging system of three linked modules of typical reinforced stainless steel two-tier laboratory cages (each module: 1.20 × 1.20 × 2.0 m, width × depth × height; total: 2.88 × 3 = 8.64 m^3), or in open-rooms (approximate total average of 22.8 m^3 per group) containing either these cages with the fronts removed or no caging at all. These different housing conditions resulted from a progressive move toward all open-room housing as part of a programme of refinement of the colony's husbandry and management (Wolfensohn 2004).

All housing conditions had a woodchip forage substrate spread on the floor. Each room contained a flexible arrangement of wooden furniture, wooden shelves and hanging items (eg tyres), had ceramic tiles or forex sheeting (Lonza Ltd, Basle, Switzerland) on the walls, protected light and electrical fittings, and a secondary door. Waste solids were removed every two days and the substrate changed weekly; rooms were thoroughly washed every 10–14 days, and cages were thoroughly washed every 7–10 days. The animals were fed three times per day: in the morning with monkey chow pellets (Harlan Teklad, Madison, Wisconsin, USA); around noon with foraging mixture (as of Davys 1995); and mid-afternoon with whole, fresh fruit. Water was available *ad libitum*. The animals were maintained under a 12:12 h light:dark lighting regime at a constant temperature (15–24°C) and humidity (45–65%), in accordance with the UK Home Office *Code of Practice for the Housing and Care of Animals in Designated Breeding and Supplying Establishments* (Home Office 1995).

Capture, sedation and blood sampling

This study used 53 adult rhesus macaques aged between 8 and 20 years: 10 from traditional cages (3 males, 7 females) and 43 from open-rooms (6 males, 37 females). These animals had been primarily raised in breeding units, having been weaned at 6–12 months and group-housed for more than 5 years. Those housed in traditional cages were sedated by injection following physical restraint using a squeeze-back mechanism; those in open-rooms were either trained to stand and present their hind-quarters for injection when a technician entered the room (Wolfensohn 2004), or were caught and physically restrained using a net before being injected.

Sedation was via an intramuscular injection of ketamine hydrochloride at a concentration of 100 mg ml^{-1}, and at a dose of 10 mg kg^{-1} (Ketaset, Fort Dodge, USA). Blood samples were obtained during routine health screening carried out under the Veterinary Surgeons Act 1966 (http://www.rcvs.org.uk/Templates/Internal.asp?NodeID=89679, accessed 4 October 2005) by needle venepuncture of the femoral vein into an EDTA Vacutainer (BD Vacutainer Systems, Plymouth, UK). 30 µl of this blood was taken immediately for the leukocyte assay; the remainder was used for measuring haematology parameters (Celltac MEK–5108K: Kohden, Japan) and differential cell counts from alcohol-fixed blood smears using May-Grunwald and Giemsa stains.

Measurement of leukocyte activity

As described by McLaren *et al* (2003), luminol (5–amino–2, 3–dihydrophthalzine [Sigma A8511]: Sigma-Aldrich Company Ltd, Poole, Dorset, UK) fluoresces in the presence of oxygen free radicals and is therefore used to measure blood chemiluminescence levels. The method used in this study is based on the studies by Mian *et al* (2003) and McLaren *et al* (2003). Appropriate concentrations of luminol (10^{-4} mol l^{-1}) and the microbial product PMA (phorbol 12–myristate 13–acetate [10^{-3} mol l^{-1} and 10^{-5} mol l^{-1}] [Sigma P8139]: Sigma-Aldrich Company Ltd, Poole, Dorset, UK) were used to measure luminescence (relative light units [RLUs]) in a portable chemiluminometer (Junior LB 9509: EG & G Berthold, Germany) for 30 s, every 5 min, over a 45 min period. Three tubes were prepared for each animal's sample: an unchallenged sample (10 µl of blood and 90 µl of luminol), and two with 10 µl of blood, 90 µl of luminol and 10 µl of PMA at each of the two concentrations (high concentration [HC] = 10^{-3} mol l^{-1} and low concentration [LC] = 10^{-5} mol l^{-1}). Tubes were agitated to mix the contents; when not in the luminometer, tubes were maintained at 37°C in a water bath. Blood was challenged with two concentrations of PMA to control for any differential response to a weak or strong concentration. An unchallenged sample allowed assessment of background leukocyte activity.

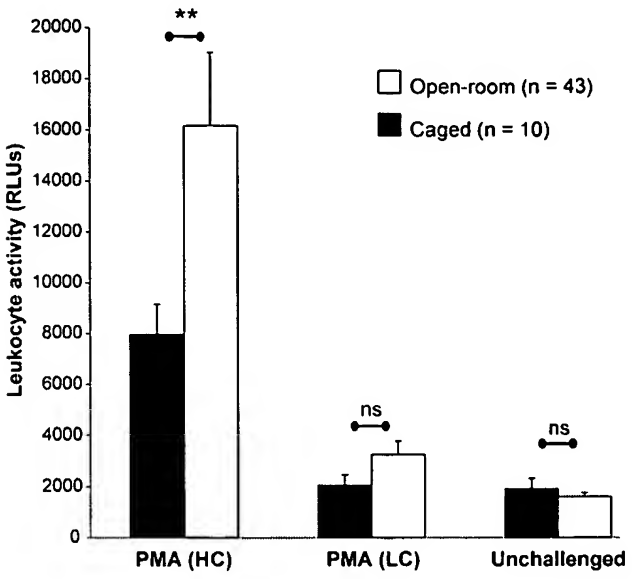
Statistical analyses

Leukocyte activity data were derived from calculating the area under the response curve and dividing by the number of neutrophils, obtained from the differential cell count, to produce a response value that reflected the total response per neutrophil. Data from the two groups (a traditional caging system versus open-rooms) were compared using the non-parametric Mann-Whitney *U* test, using SPSS 11.5® for Windows®.

Results

Comparison of the leukocyte activity of differently housed rhesus macaques revealed that macaques housed in a traditional caging system produced a significantly lower response than those housed in open-rooms to challenge with PMA at high concentration (Mann-Whitney $U = 97.0$, $P = 0.007$) (see Table 1 and Figure 1). A lower response was also found for caged monkeys with the low concentration of PMA, but this was not significant ($U = 184.0$, $P = 0.481$). There was no significant difference in background, unchallenged activity ($U = 190.0$, $P = 0.570$) (see Table 1), and no significant differences were found between the two housing types in the number or composition of leukocytes (see Table 1).

Figure 1



Leukocyte activity in blood challenged with two different concentrations of PMA (HC: 10^{-3} mol l^{-1}; LC: 10^{-5} mol l^{-1}) in rhesus macaques housed in a traditional caging system versus open-rooms. Bars represent the mean total leukocyte activity (+ standard error of the mean) over 45 min, divided by the number of neutrophils (10^9 l^{-1}); ns = non significant, ** = $P < 0.01$.

As this result could be confounded by differential response to capture technique, ie standing for injection versus physical restraint (net or squeeze-back mechanism), the data from open-room-housed animals, where the capture method (netting or standing) was known, were tested. No significant difference was observed in the leukocyte response to challenge with PMA (see Table 2).

Conclusions

There is considerable evidence to suggest that stress may reduce the effectiveness of the immune system, therefore increasing the risk of infection or disease (Dhabhar *et al* 1995; Kang *et al* 1996; Raberg *et al* 1998). Research suggests that even short-term stress can produce demonstrable and immediate physiological changes in heart rate, blood pressure and the activation of leukocytes (Ellard *et al* 2001; Mian *et al* 2003); these changes depend on the nature and intensity of the stressor (Willard *et al* 1989; McLaren *et al* 2003). In humans, epidemiological studies support the idea that individuals who are more stressed have a suppressed immune system (eg Graham *et al* 1986); stress has also been linked to disease in farm animals (eg Koolhaas *et al* 1999). Therefore, a reduction of immune reactivity as an indicator of stress is not a novel observation (Murata 1989; Haigh *et al* 1997; Maes *et al* 1998). Indeed

McLaren *et al* (2003) recently reported that in the absence of other underlying pathology an alteration the leukocyte activity is a good indicator of stress.

The animals used in this study were of an extremely high health status, being part of a specific-pathogen-free colony, where they were subjected to regular health screening and continuous surveillance by trained staff. These screenings indicated that there was no difference in the underlying pathology between the treatment groups. All other parameters for the treatment groups were the same as they were housed under regulated conditions and received the same nutrition. This, coupled with the evidence of accommodation size-related stress (see Caging and stress), and clear differences in the space provision in this study, strongly supports that a reduction in the leukocyte responsiveness is an indicator of differences in stress levels, as supported by the published literature (eg Willard *et al* 1989; McLaren *et al* 2003; Mian *et al* 2003; Montes *et al* 2004; Mian *et al* 2005).

This study shows that the leukocyte activation test is appropriate for the measurement of stress in non-human primates, specifically in rhesus macaques. Lower leukocyte response to *in vitro* PMA challenge in animals housed in a traditional caging system, compared with open-room housed animals, demonstrates that cage housing is associated with higher levels of physiological stress. Results also show that this effect was not affected by whether individuals were physically restrained or trained to stand for a sedative injection. A significant difference in the response of the treatment groups to only one of the two PMA concentrations suggests that the leukocyte population may contain functionally different types of neutrophils, responding to different levels or nature of challenge. Where those types that respond to less stressful stimuli have already been used, a further mild challenge may not result in a significant response. Eliciting a further response may, as in this case, require a more powerful stimulus, eg a higher concentration of PMA. The composition of the neutrophil population by type may vary following an acute stressor or as a result of living in chronically stressful conditions.

Unlike previous studies using this technique (ie McLaren *et al* 2003; Montes *et al* 2004), differences in leukocyte activity between the treatment groups were not accompanied by differences in leukocyte number or composition. This may be attributable to differing demographics of the leucocyte populations in the two treatment groups. Stressed animals may be recruiting more immature leukocytes (ie band neutrophils) to replace those used in the stress response, which are represented in cell counts but are not sufficiently mature to emit superoxide in response to a challenge. Therefore, more and less stressed animals may have the same leukocyte population sizes, but more stressed animals may have a greater proportion of neutrophils that are too immature to respond to a further challenge; therefore the total neutrophil response would be less.

Animal welfare implications

This study provides evidence that open-rooms are less stressful than a traditional caging system for groups of rhesus macaques. This leukocyte activation test can be used to indicate the immune competence of animals, highlighting any requirement for additional health monitoring or modification of management timetables, which may include further potentially stressful events (eg change in group composition, weaning of offspring etc). The combination of additional veterinary attention and deferral of further stressful events will optimise the physical and psychological health of an animal, which may otherwise be compromised by compounding an already heightened stress level. This has implications for the animal's reproductive potential as well as its value as an experimental model, as high stress levels are well known to negatively impact on both these factors.

The leukocyte activation test also allows the measurement of physiological stress using much smaller blood samples than for traditional blood cortisol measures (30 µl versus approximately 2 ml). This small amount can be obtained by capillary sampling from a heel prick on trained animals, avoiding the need for sedation and the possible negative consequences (eg haematoma, phlebitis) associated with needle venepuncture sampling. This technique confirms the refinement of housing in the study colony, allows refinement of the management of the animals, and is conducive to the refinement of the sampling technique, with a decrease in blood sample size to approximately 30 µl.

It should, however, be remembered that this technique may not be practical in some situations, for example, in some zoos and sanctuaries where blood sampling may be acceptable for veterinary/diagnostic purposes but not for research purposes where, in the UK, a Home Office licence would be required.

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